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14. ABSTRACT

The objective of this study is to develop a protein that can be injected directly into the eye while in the combat theater to prevent or reduce the damage to retinal cells following blast associated trauma. To this end, we propose use of a peptide (POD, peptide for ocular delivery) that we have previously shown to be capable of penetrating retinal cells in vivo. POD will be utilized to deliver melanoma inhibitor of apoptosis (ML-IAP) or X-linked inhibitor of apoptosis (XIAP) into retinal cells, neither of which have a cell penetrating capacity by themselves. We are using recombinant adenovirus as the gene expression system to synthesize the POD fusion proteins. At the end of Year 1 of this proposal, we have completed the construction of 1 (XIAP) of the recombinant adenovirus constructs and are at the penultimate step of generating the ML-IAP construct. Next steps are to purify these two proteins and evaluate their anti-apoptotic properties in vitro and ultimately in vivo. During the following year we will complete the construction of the ML-IAP construct and relevant controls and perform studies to test the anti-apoptotic properties of these recombinant proteins.

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Table of Contents

Platform for Rapid Delivery of Biologics and Drugs to Ocular Cells and Tissues Following Combat Associated Trauma

ANNUAL REPORT 2014

ABSTRACT

The aim of this study is to develop a novel therapeutic to prevent retinal degeneration upon blast trauma. Such therapeutic will be a fusion protein, consisting of the POD domain for ocular delivery and an anti-apoptotic protein or protein domain, respectively, such as X-linked inhibitor of apoptosis (XIAP), melanoma inhibitor of apoptosis (ML-IAP) or the Ku70 V5 domain. For large scale production of these respective proteins, we are developing eukaryotic expression vectors, specifically recombinant adenovirus vectors. During the previous year, we have made significant progress in our studies. Specifically, we have completed the synthesis of POD-XIAP and control viruses and demonstrated that they can produce high levels of recombinant protein in eukaryotic cells. We are currently developing protocols for optimal protein purification. More exciting is our preliminary data indicating that Ku70 V5 can inhibit apoptosis in a mouse model of retinal apoptosis. Contrary to our initial expectation, our results indicate that not only is the peptide efficacious after intravitreal delivery (a standard of clinical care) but also when delivered topically to the eye. These exciting preliminary results take us beyond the initial scope of the planned study and open new opportunities of investigation. Below we present results on our efforts to purify POD-XIAP and XIAP (negative control) and include in preliminary in vivo data on the use of V5 to inhibit retinal apoptosis.

INTRODUCTION

Blast injury induces several cell death pathways including apoptosis. Retinal ganglion cells and photoreceptors are not capable of self-renewal and loss of retinal neurons leads to impaired vision or complete blindness. In order to preserve vision prior to it being irreversibly irreparable, it is imperative to block cell death pathways such as apoptosis subsequent to acute trauma, ideally by self-administration of a drug or a biologic that can be included in the medical kit. Developing a platform technology and proving its usefulness in at least one pathway, i.e. apoptosis, is the major goal of this proposal. The peptide for ocular delivery (POD) has been validated for efficient transport of DNA and proteins to the eye. Since expression from DNA is generally slow (12+ hours), our goal is to develop a protein that could act immediately (within minutes) upon injection prior to the onslaught of the damage created by apoptosis. We envisage a lyophilized product provided in the medical kit that the member of the armed forces could re-suspend in packaging and request a colleague to inject into the eye or self administrate if necessary. In this study, several different adenovirus constructs will be developed to generate recombinant proteins - POD fusions with the anti-apoptotic proteins 'melanoma inhibitor of apoptosis (ML-IAP) and X-linked inhibitor of apoptosis. The purified proteins will then be evaluated in vitro and in vivo for their anti-apoptotic and thereby protective capacities. We will utilize a blue light induced model of apoptosis in mice, a model system that has been well characterized in our laboratory and intended to act as a surrogate for trauma associated with any event that triggers apoptosis such as blast wind, radiation, heat etc. In parallel, we will investigate the potential use of an inhibitor of apoptosis known as Ku70 V5, which as indicated below, has already proven effective in our preliminary results described below.

RESULTS

POD-fusion proteins and control adenoviruses

In our previous (2013) Annual Report we described that expression of POD-fusion proteins was below optimal levels and Ad-XIAPhis control viruses did not efficiently reproduce in 911 or produce significant levels of recombinant proteins in ARPE-19 cells. As modifications of the infection protocol did not increase efficiency, new constructs of the respective viruses were generated. The new constructs – both the XIAPhis viruses (POD fusion and control) exhibited efficient cytopathic effect (CPE), a sign of virus propagation (see Figure 1 for Ad1-POD-XIAPhis).
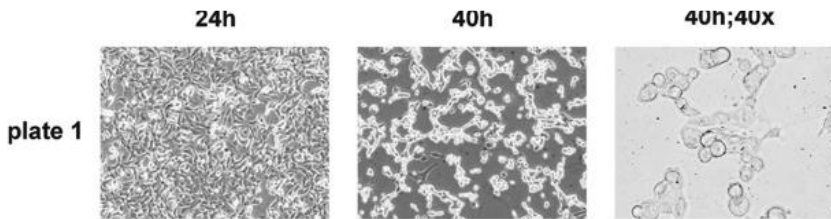

Figure 1: CPE in 911 cells at 24h and 40h after infection with Ad1-POD-XIAPhis clone 4.
Rounding up of cell and loss of surface adherence as hallmarks of CPE. Right column shows virus producing cells at a higher magnification.

ML-IAPhis constructs didn't exhibit CPE. Hence, we decided to focus on the expansion of the XIAP viruses and to proceed to next steps of this study. We generated a total of 5ml of purified virus per clone, considered sufficient for all subsequent experiments.

POD-XIAPhis & XIAPhis protein expression

In western blot analyses of protein expression in ARPE-19 cells, we found that both XIAPhis viruses seemed to synthesize protein. However, it appeared that the POD fusion virus was more efficient (Figure 2).
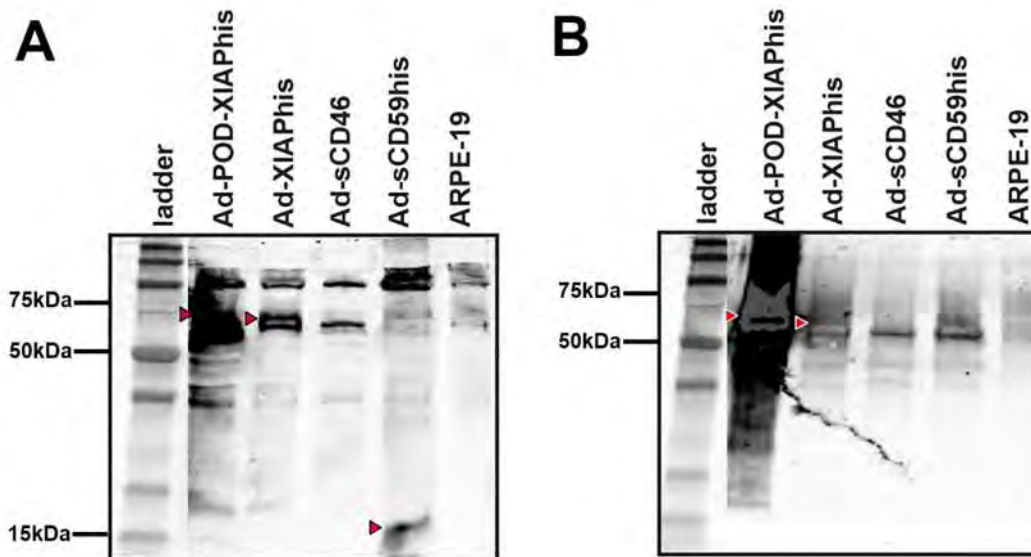

Figure 2: Western blot (WB) analysis
(**A**) Using an updated anti-6x-his antibody, unique bands were detected in ARPE-19 cell that were infected with the Ad1-POD-XIAPhis virus (expected size 61.7kDa), the Ad1-XIAPhis virus (expected size 57.5kDa) or the Ad1-sCD59his virus (expected size 15kDa) that served as a positive control for his-detection. Specificity was assumed by size compared to the ladder and absence in uninfected wild-type (wt) ARPE-19 cells and ARPE-19 infected with an Ad1 virus coding for a non-his-tagged sCD46 protein. (**B**) Detection of the same samples with a XIAP-specific showed unique bands in samples from Ad1-POD-XIAPhis and Ad1-XIAPhis infected ARPE-19, all other infected cells or non-infected wt cells showed nonspecific background detection. Comparison of band intensities indicated stronger expression from the POD-XIAPhis virus compared to the XIAPhis virus.

To further investigate expression of the protein in living cells, we performed immunofluorescence stainings of POD-XIAPhis and XIAPhis proteins in infected ARPE-19 cells using specific antibodies either directed against the 6xhis-tag or human XIAP for detection.
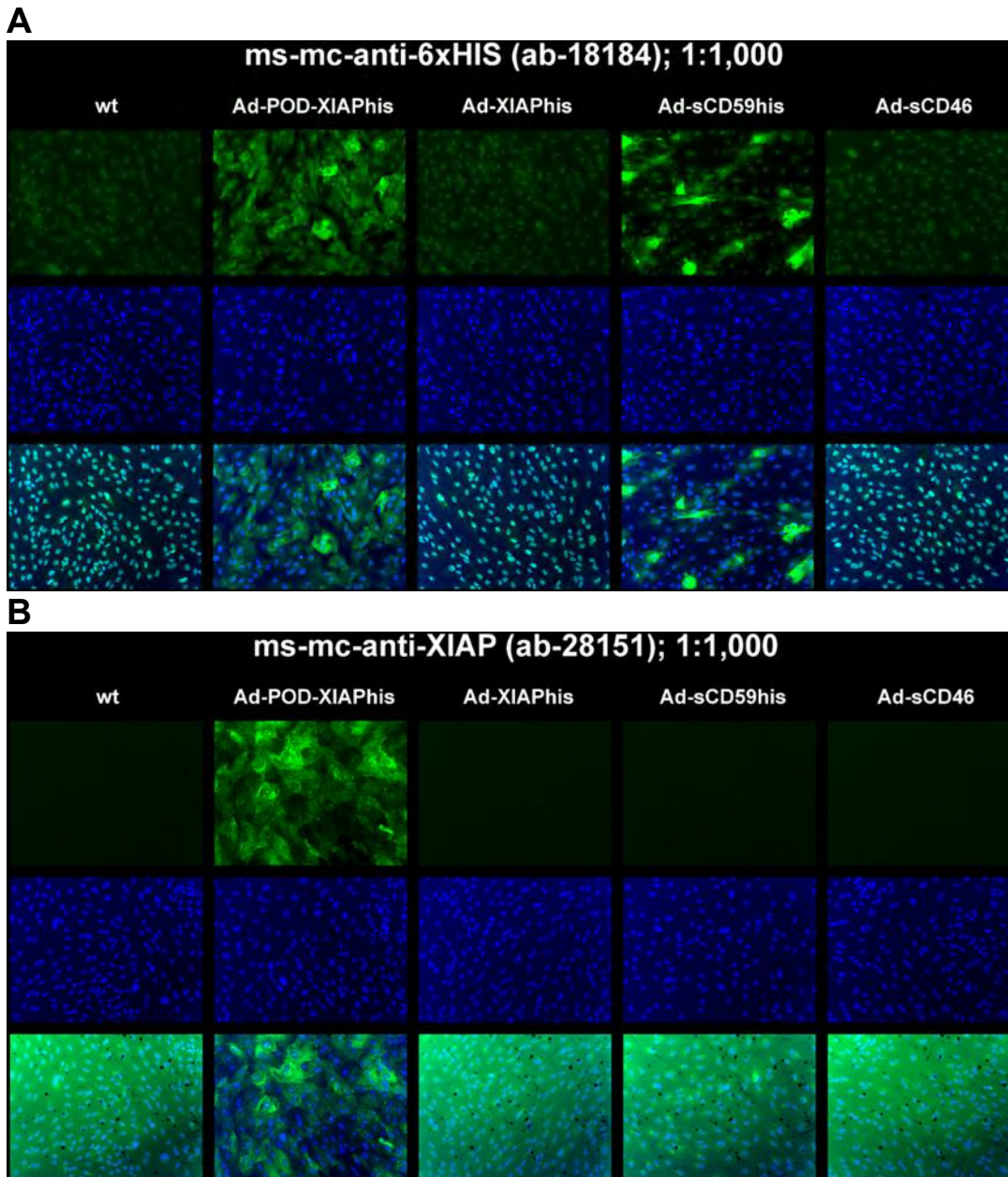
Figure 3: Immunofluorescence (IF) stainings
(**A**) Using the anti-6x-his antibody, signals were detected only in ARPE-19 cells that were infected with the Ad1-POD-XIAPhis virus or the Ad1-sCD59his control virus. Uninfected wild-type (wt) ARPE-19 cells and ARPE-19 infected with an Ad1 virus coding for a non-his-tagged sCD46 protein showed no labeling. Notably, we did not detected signals in Ad1-XIAPhis infected ARPE-19. (**B**) Detection utilizing a XIAP specific antibody resulted in a clear signal exclusively in Ad1-POD-XIAPhis infected cells, all other infected cells or non-infected wt cells were not labeled. We concluded that the new POD-XIAPhis virus is productive.

We observed strong signals with both antibodies in Ad-POD-XIAPhis infected cells, but detected no signals in cells infected with the Ad1-XIAPhis control virus (Figure 3). Moreover we found that the POD-fusion protein was located mainly in the cytoplasm of the cells (Figure 4), an important observation as it influenced the choice of the buffer used for cell lysis and purification.
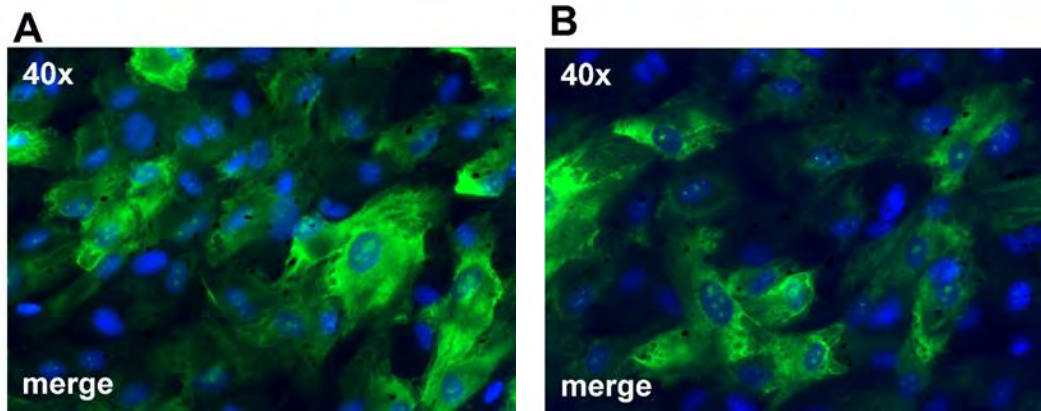
Figure 4: Immunofluorescence (IF) stainings for intracellular localization
Signals detected with the anti-his antidody (**A**) and the anti XIAP antibody (**B**) indicated localization of the POD-XIAPhis protein throughout the cytoplasm and within the nucleoli, sub-nuclear sites of high expression activity. Nuclei are visualized by DAPI-staining.

We conclude that only the POD-XIAPhis virus is functional and produces the corresponding protein efficiently. It is known that protein yield from adenoviruses differs depending on the host cell line, thus we tested expression in ARPE-19, 293T and 911 cells. 293T and 911 cells are replicating cells and therefore expected to produce higher amounts of protein. However, these cells are sensitive to infection and during protein production, we observed significant cell death. ARPE-19 cells, in contrast, are very stable in this respect, but expected to be less effective in protein synthesis as they are non-replicating cells. Our results indicated highest efficiency in 293T cells followed by 911 cells. Thus all further infections for protein expression were performed in 293T or 911 cells. The following approach focused on the choice of lysis buffer. Here we tested the commercial CelLytic M lysis Buffer (Sigma) versus a freeze/thaw lysis in a Tris-HCl based buffer.
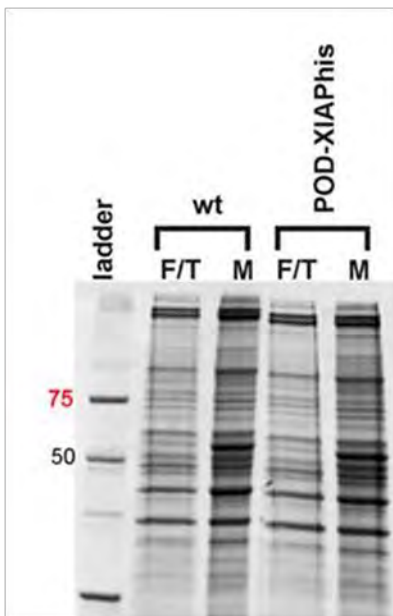

Figure 5: Lysis buffer comparison
Coomassie stained SDS-gel of 911 cell lysates generated by lysis with the CelLyticM lysis buffer (M) or freeze/thaw lysis (F/T) in a Tris-HCl based buffer.

Based on these results we decided to use the CelLyticM buffer, as the overall protein content appeared higher in these samples (Figure 5).

Next we compared the binding capacities of different matrices for the POD-XIAPhis protein. We tested Ni^{2+}- and Co^{2+}-loaded agarose beads which target the 6xhis-tag and Heparansulfate loaded agorose beads, as the POD portion has a heparin-binding capacity.
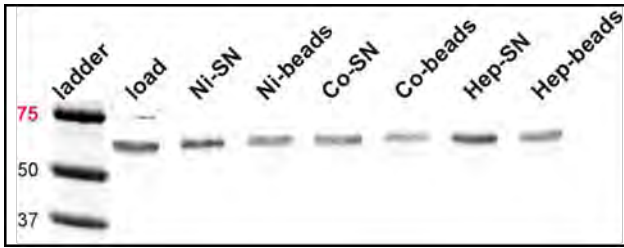

Figure 6: Binding efficacy test
WB-detection of POD-XIAPhis with a HIS-specific antibody in the supernatants and the sedimented bead-fractions after incubation of clear lysate of POD-XIAPhis infected ARPE-19 cells (load) with Ni^{2+}-beads, Co^{2+}-beads or Heparin-beads.

The overall results of these pull-down experiments was that both HIS binding matrices worked at a similar efficiency. The Heparin beads also bound the protein, the efficacy, however, appeared lower (Figure 6).

At that stage we designed a protocol using CelLyticM buffer for cell lysis and a Co^{2+}-loaded chromatography column for the first large scale purification approach.
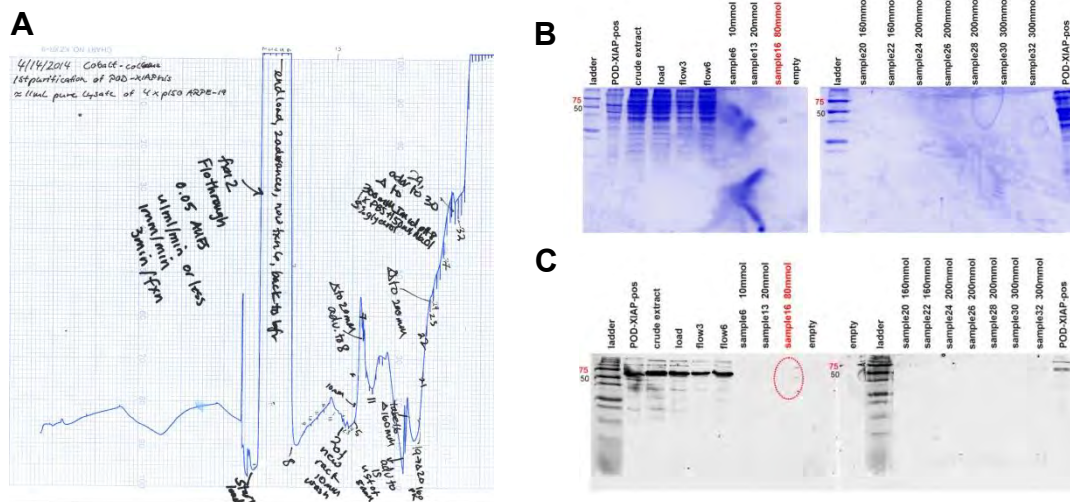

Figure 7: First Co^{2+}-column purification of POD-XIAPhis
(**A**) 10mL of clear CelLytic M lysate from ARPE-19 cells infected for 4d with Ad-POD-XIAPhis were loaded onto a 1mL Co^{2+}-chromatography column at a constant flow rate of 1mL/min^{-1} and 1bar pressure. AUFS were constantly recorded and flow-through, wash- and elution fractions were automatically collected in a fractionator. Protein elution was achieved by increasing the imidazole content in the flow buffer in 20mM steps from 0 to 300mM. (**B**) Coomassie stained SDS-PAGE of column fractions (**C**) WB detection utilizing a XIAP specific antibody of the fractions shown in (B).

Analysis of the collected fractions by Coomassie staining and WB indicated that there was no protein binding to the Co^{2+}-column as the flow-through fractions had the highest protein contents (Figure 7B, C). A 61.7kDa corresponding to POD-XIAPhis was not detected in wash or elution fractions (Figure 7C).

Based on this result we switched to the use of a Tris-HCl based buffer system in combination with freeze/thaw-lysis. Additionally we switched the column to a Ni^{2+}-charged system. All other setting were kept constant, the lysate was generated as previously described.
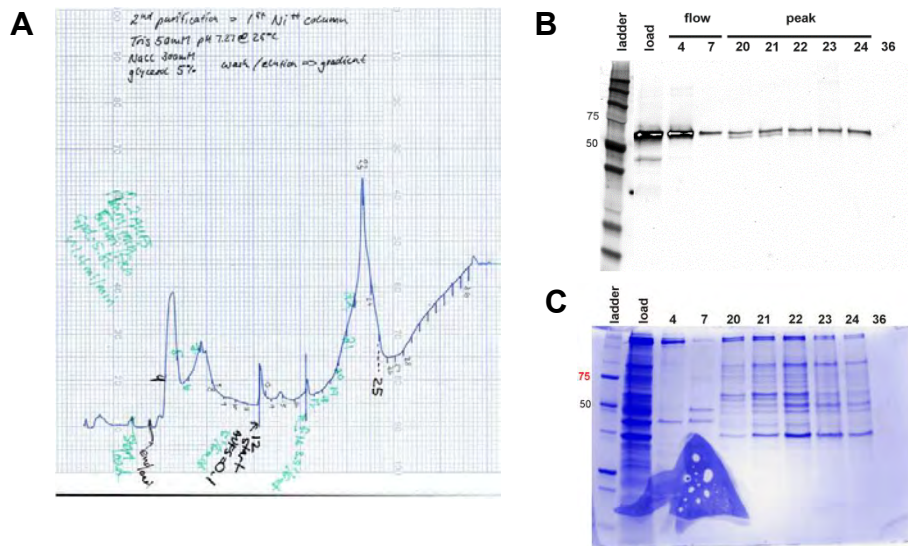
Figure 8: First Ni^{2+}-column purification of POD-XIAPhis
(**A**) 10mL of clear lysate from ARPE-19 cells infected for 4d with Ad-POD-XIAPhis in 50mM Tris-HCl, 300mM NaCl, 5% glycerol pH7.27 were loaded onto a 5mL Ni^{2+}-chromatography column at a constant flow rate of 1mL/min^{-1} and 1bar pressure. AUFS were constantly recorded and flow-through, wash- and elution fractions were automatically collected in a fractionator. Protein elution was achieved by increasing the imidazole content in the buffer in a linear gradient from 0 to 300mM. (**B**) WB detection utilizing a XIAP specific antibody in the column fractions (**C**) Coomassie stained SDS-PAGE of column fractions.

The UV-graph (Figure 8A) showed a distinct peak coming off the column at an imidazole concentration of about 90mM spanning fractions 20 to 24. POD-XIAPhis protein was detected in these peak fractions using a anti-XIAP specific antibody in western blot experiments (Figure 8B). However, a band of the same size 61.7kDa was also detected in wash fractions coming off at lower imidazole concentrations (not shown) and still was detected at highest intensities in the flow through. Analysis of samples of these fractions by Coomassie staining also indicated that the peak fractions still contain significant amounts of background impurities (Figure 8C).

We concluded that the second approach provided a principally working purification system that admittedly requires improvement.

Currently approaches to include the Heparin binding capacity provided by the POD fusion are being examined. Moreover the benefit of size separation chromatography is tested as a second step of purification.

In vitro assay for anti-apoptotic effects of purified POD-XIAPhis fusion proteins
To assess the functionality of the purified proteins before application in animals, we wished to establish a cell based in vitro assay. Staurosporine (STS) from *Streptomyces staurospores* is a non-selective protein kinase inhibitor and frequently used as a general inducer of apoptosis. In a pilot experiment, ARPE-19 cells were incubated with different concentrations of STS (0.1/1.0/5.0μM) over night.
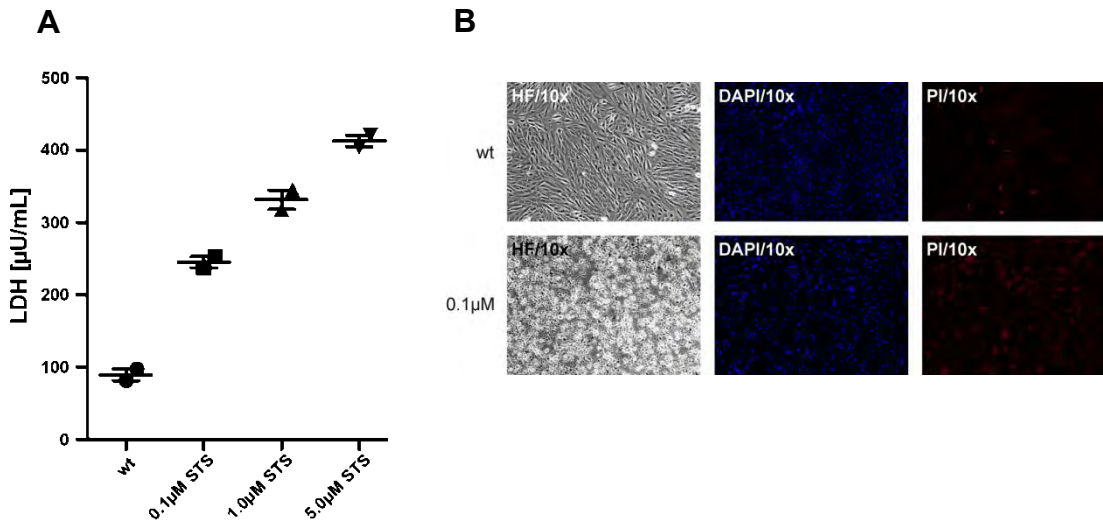
Figure 9: In vitro anti-apoptosis assay
ARPE-19 cells were incubated overnight with 0 to 5µM STS. (**A**) Plot of the LDH-assay quantifying the LDH in the cell medium. (**B**) Bright field micrographs (left panels) demonstrating cell morphology of ARPE-19 after incubation with 0µM STS (wt) and 0.1µM STS overnight. Middle panels show DAPI counterstained nuclei of the cells, right panels demonstrate PI uptake into the cells.

The degree of cell degeneration was quantified as the amount of LDH in the medium, which increases, when cell membranes disintegrate during apoptotic degeneration. Additionally, uptake of PI was visualized as a graphic marker for cell membrane disintegration. Our data indicate that 0.1µM STS increased the LDH release by 2.5fold compared to wt ARPE-19 cells (Figure 9A) and that these cells showed a distinct increase in PI uptake (Figure 9B). Higher concentrations of STS led to an even further increased LDH release (Figure 9A), the cells, however, were almost completely dead at these concentrations determined by PI and DAPI staining (not shown). Based on these results we choose 0.1µM STS as the working concentration. The optimal incubation time is currently determined in ongoing experiments.

Anti-apoptotic POD fusion protein POD-V5
During the optimization of the adenovirus based protein expression/purification system, we examined an additional POD fusion protein with anti-apoptotic properties. This fusion combines the POD delivery ability with the reported anti-apoptotic capacity of the V5 protein domain of the Ku70 protein. To test this fusions effect in vivo, apotosis was induced in the retinas of balb/c albino mice by exposure to blue light, a standard model for retinal apoptosis. Different concentrations of purified POD-V5 protein were intravitreally injected into the eyes subsequently. The corresponding control mice received ocular injections of phosphate buffer saline (PBS), the solvent of POD-V5. As a measure of the degree of apoptosis the thickness of the layer of cells protected from blue light within the outer nuclear layer (ONL) of the retina, the main target of blue light induced apoptosis, was determined by optical coherence tomography (OCT) 48h post injection.
While the PBS-controls displayed a conspicuous thinning of this layer we observed that injection of 1nmol POD-V5 inhibited this thinning (Figure 10A). Quantification revealed an average thickness for the layer of protected cells of 29.58 ± 2.95 in these animals, compared to an average of 16.48 ± 1.86 in the controls. Statistical analysis indicated a significant level of protection (*p=0.0037*; n=6; Figure 10B).
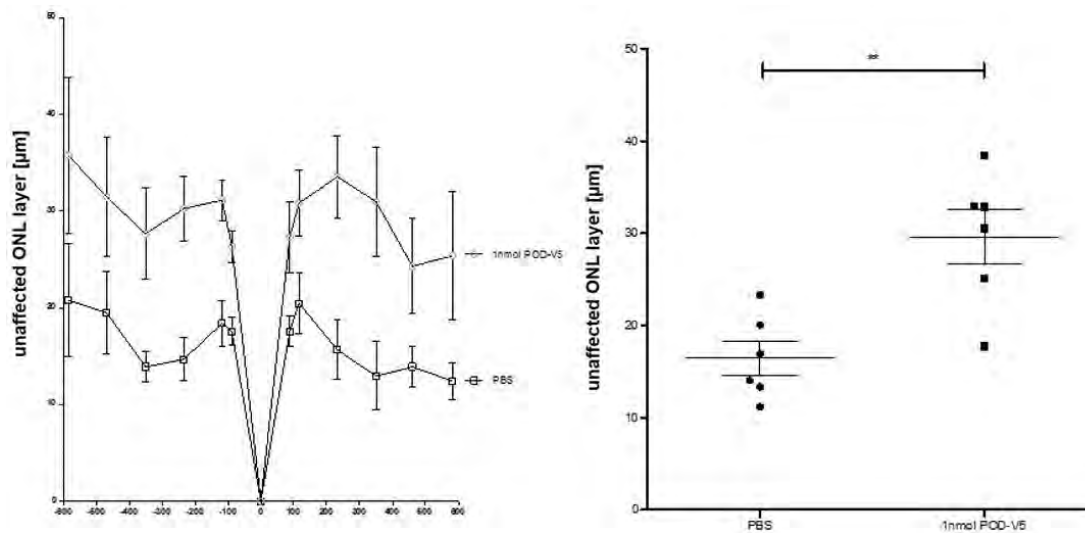
Fig. 10: OCT measurement of ONL thickness in balb/c eyes injected with 1nmol POD-V5 and PBS.
PBS injected eyes showed a significant reduced thickness of the ONL. In contrast, injection of 1nmol POD-V5 efficiently protected from ONL thinning. Left side: ONL thickness vs. distance from the optic nerve (0); Right side: single values and mean average ± SD. **: $p \leq 0.005$.

Moreover, our experiments indicated the existence of a limiting concentration as injection of a higher concentration (10nmol) as well as of a lower concentration (0.1nmol) of POD-V5 had no protective effect against blue light effects in the ONL (Figure 11).
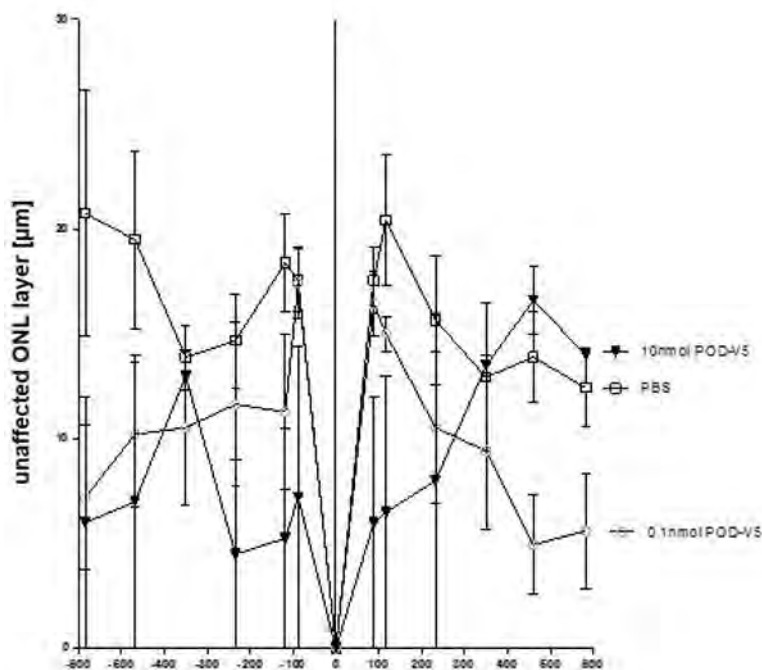

Fig. 11: OCT measurement of the layer thickness of unaffected cells in balb/c eyes injected with 10 and 0.1nmol POD-V5 and PBS.
Neither 10nmol nor 0.1nmol POD-V5 protected from layer thinning as demonstrated by the thickness plot.

We concluded that POD-V5 is an efficient tool to inhibit apoptotic processes in the retina when intravitreally injected even after the initial damage (blue light) has already begun. We further analysed the contribution of POD to the protective V5-effect. Therefore V5 was intravitreally injected at equimolar amounts (1mmol) to allow direct comparison to POD-V5.
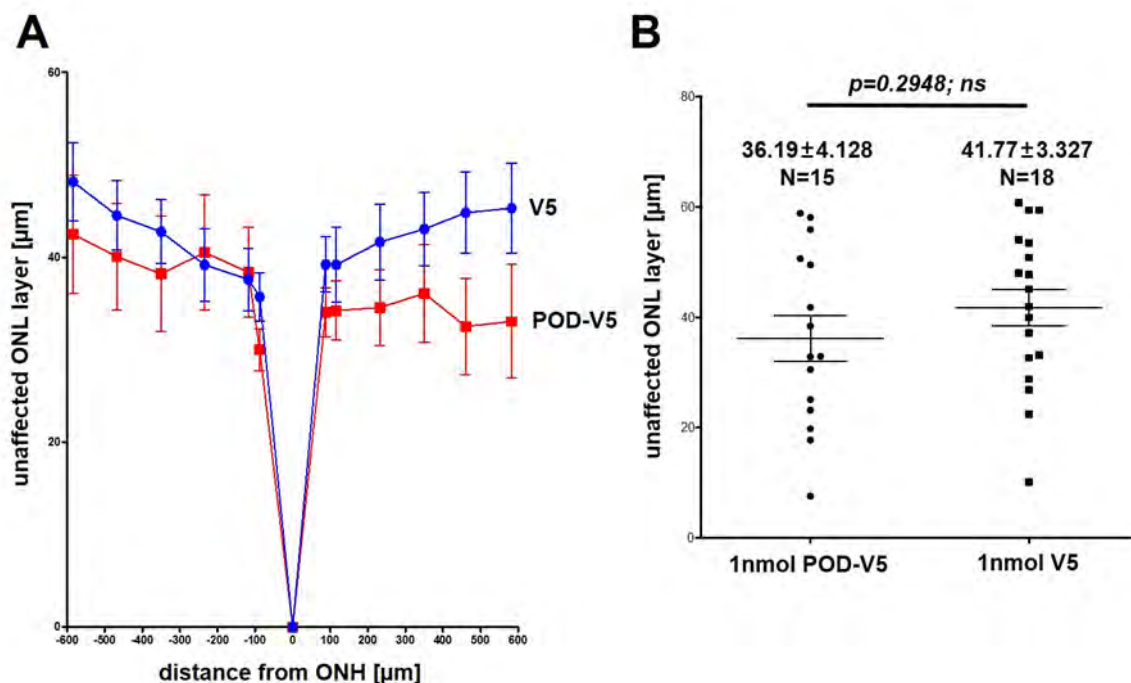
Figure 12: Comparison of protective effects of POD-V5 and V5
1nmol POD-V5 and V5 were intravitreally injected immediately after exposure to blue light. Thickness of the protectet layer was measured by OCT. (**A**) Retinogram plotting thickness of protected ONL layers against the distance from the optin nerve. (**B**) Mean averages ± S.D. of layer thicknesses in POD-V5 and V5 injected eyes, respectively. The p-value of 0.2948 indicates no significant difference between the two treatment groups.

Based on these data we concluded that POD did not alter the protection from apoptotic degeneration mediated by V5. At this point we decided to proceed with the V5 peptide and test it in topical applications. Preliminary results based on OCT data indicate a protective effect at 200nmol V5 (Figure 13).
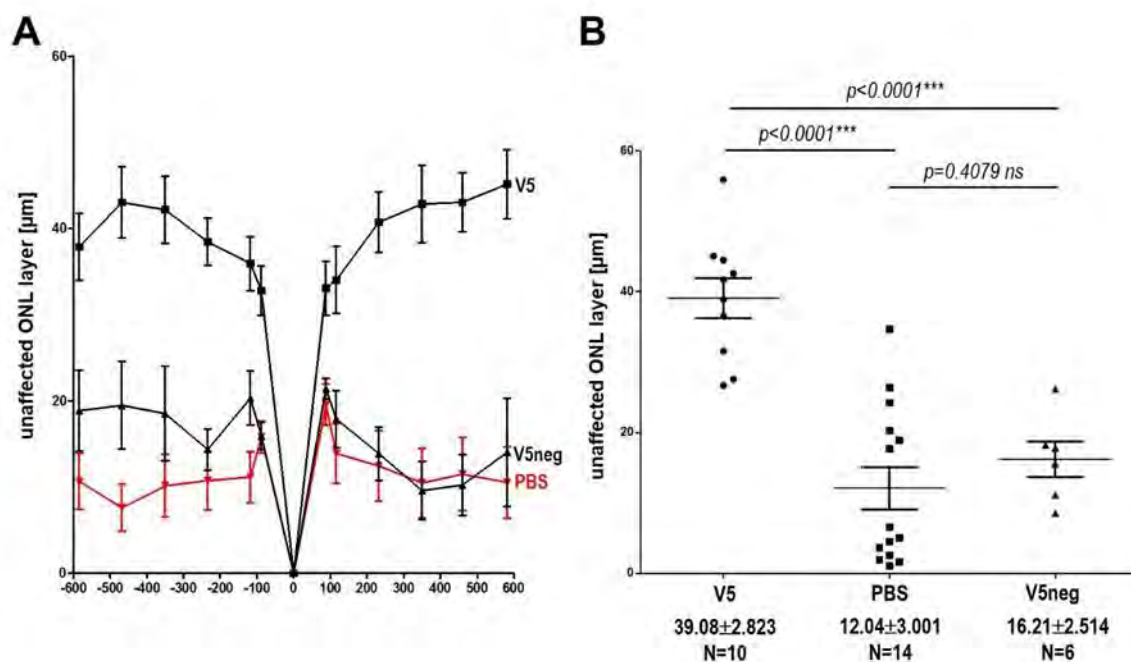

Figure 13: Protective effect of topically applied V5
200nmol V5 and V5neg (cell penetrating but not anti-apotptic) were topically applied twice one day before blue light, once immediately after blue light and twice one day after blue light. PBS was applied analogous as a solvent control. 2 days after blue light, thickness of the protectet ONL layer was measured by OCT. (**A**) Retinogram plotting thickness of protected ONL layers against the distance from the optin nerve. (**B**) Mean averages ± S.D. of layer thicknesses in V5, V5neg and PBS treated eyes, respectively. The p-values of <0.0001 indicate a highly significant difference between the V5 group and the V5neg and PBS groups.

12

Histological analysis proved that this protection is associated with the anti-apoptotic effect of V5. One classical marker of apoptosis is DNA fragmentation, which can be visualized by DNA labeling in TUNEL assays (Figure 14).
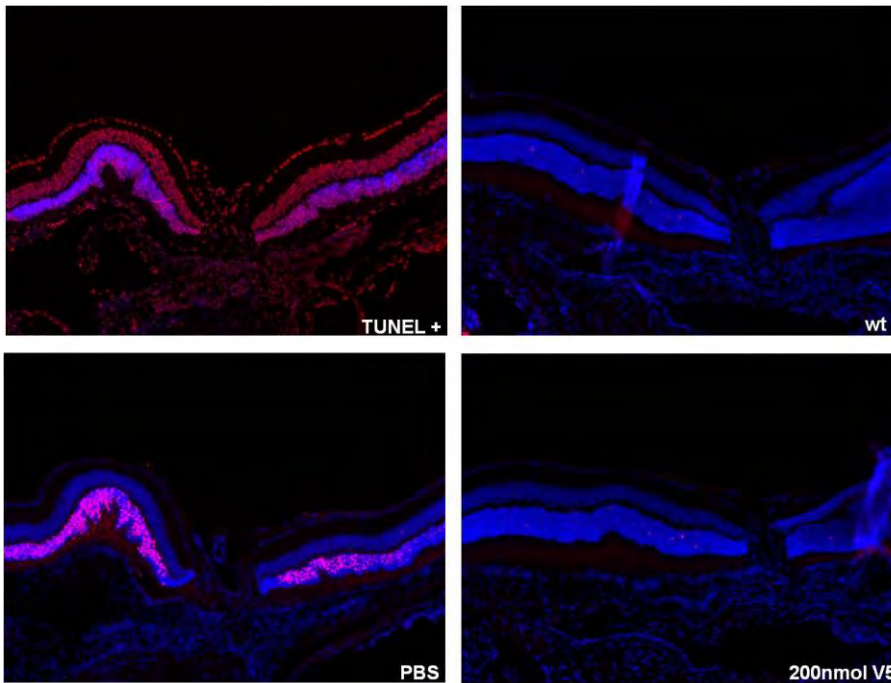

Figure 14: TUNEL assay
Left upper panel: TUNEL assay control showing DNA fragmentation (red) over the entire section. Right upper panel: section of a wt eye showing no signs of apoptosis. Left lower panel: section of an eye treated with the solvents control PBS showing increased TUNEL positive cells in the ONL around the optic nerve after blue light exposure. Lower right panel: section of an eye treated with 200nmol V5 showing only very sparse TUNEL positive cells in the ONL around the optic nerve after blue light exposure.

We conclude that V5 effectively reduces apoptotic ONL degeneration when applied topically to the eye.

ISSUES

In our initial attempt we failed to recover the desired recombinant clones but we have solved the technical issues and some of the desired clones have now been rescued. Overall, the project is in line with the schedule defined in the statement of work (SOW).

KEY RESEARCH ACCOMPLISHMENTS

- New shuttle plasmids for all viruses were genereated
- The Ad1-POD-XIAPhis was generated, purified and proven functional
- Different protein purification systems were tested
- An in vitro apoptosis assay was established
- Topical application of the anti-apoptotic V5 peptide protected from retinal apoptosis in vivo

CONCLUSION

Aims defined in the SOW for year one of the project were accomplished. Production and purification of Ad1-XIAPhis, Ad1-POD-ML-IAPhis and Ad1-ML-IAPhis is currently in progress, functional viruses are expected to be generated end of 2014/ early 2015. Purification of sufficient amounts of POD-XIAPhis protein for in vivo eperiments is expected early 2015.

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SUPPORTING DATA
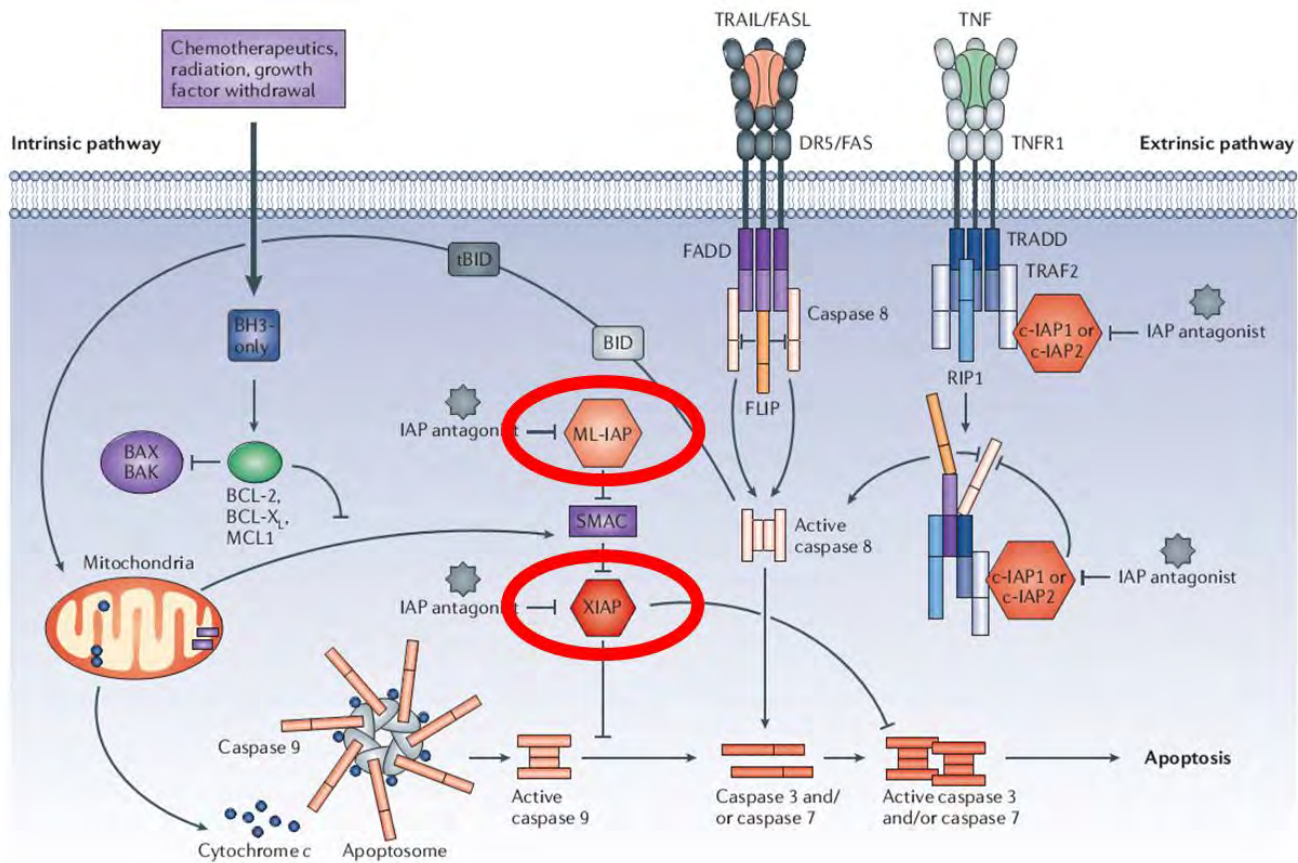
(modified from Ref. 6: Fulda S, and Vucic D: *Targeting IAP proteins for therapeutic intervention in cancer*. Nat Rev Drug Discov, 2012;11(2):109-24.)

Supporting Figure 1: Functions of MLIAP and XIAP in the process of apoptosis

XIAP inhibits the activation of pro-caspases 3 and 7 by caspase 9 in the intrinsic pathway of apoptosis. Moreover it blocks active caspases 3 and 7 activated by caspase 8 in the extrinsic pathway (FAS and TNF elicited). MLIAP blocks the XIAP inhibitor SMAC (DIABLO), which is released from mitochondria when the intrinsic pathway of apoptosis is activated.